Nucleic Acids Research, Vol. 20, No. 7 1675–1678

# Parallel analysis of oligodeoxyribonucleotide (oligonucleotide) interactions. I. Analysis of factors influencing oligonucleotide duplex formation

Uwe Maskos and Edwin M.Southern
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

Received December 12, 1991; Revised and Accepted March 9, 1992

## ABSTRACT

**A novel method for the analysis of oligonucleotide–oligonucleotide interactions is described. Oligonucleotides of different sequence are synthesised *in situ* as stripes on the surface of a glass slide (see accompanying paper). Multiple hybridisations are then carried out on each oligonucleotide simultaneously to determine the dependence of oligonucleotide duplex formation on duplex length, base composition, hybridisation solvent and sequence complexity.**

## INTRODUCTION

The advent of the PCR (1) and the widespread use of synthetic oligonucleotides as hybridisation probes (2) have rekindled an interest in the factors determining oligonucleotide duplex formation, e.g. under conditions far from thermodynamic equilibrium, which is generally the case in those applications. It is increasingly necessary to find hybridisation conditions that optimise the yield of perfect duplex and minimise non-specific hybridisation.

Previous analyses of oligonucleotide–oligonucleotide interactions have been confined to carrying out one or a few analyses at a time, with two oligonucleotides interacting in solution (3), or by immobilising one oligonucleotide on a solid support, e.g. cellulose in a column (4), or several oligonucleotides on a membrane (5, 6).

For extensive studies of DNA hybridisation it would be highly desirable to carry out analysis of many different sequences at the same time under identical conditions, i.e. concentration and amount of immobilised and free oligonucleotide, temperature and time. These are usually very difficult to reproduce in column or solution experiments. Filter hybridisations have the potential to immobilise many different sequences, but there can be substantial loss of the oligonucleotides if they are not covalently bound.

Our strategy is to synthesise many different oligonucleotides on the surface of glass slides and then carry out parallel hybridisations with test oligonucleotides. *In situ* synthesis ensures uniformity of yield of the oligonucleotide and the covalent attachment to the surface ensures stability during repeated hybridisations (see accompanying paper). Carrying out hybridisations to all the different sequences on the same plate in a single experiment results in identical experimental conditions for every individual sequence.

## MATERIALS AND METHODS

### Synthesis of oligonucleotides on slides

Standard glass microscope slides (BDH, 76 mm×26 mm) were derivatised with a linker molecule as described in the accompanying paper. Standard H-phosphonate chemistry and conditions were used for the synthesis (7).

In order to apply the coupling solution to the surface of the derivatised slide, a synthesis 'mask' was prepared: Parallel lines of silicone rubber tubing (1 mm outer diameter, Altec Ltd.) were glued to the surface of a 25 cm×25 cm glass plate with silicone rubber cement ('CAF4', Rhône-Poulenc) at 3 mm intervals.

The outline of each synthesis step was as follows: The microscope slides were placed onto the mask with their derivatised sides down and held in position by clamping with fold-back paper clips. The channels formed by the silicone rubber tubing were gassed extensively with argon. Two syringes, one containing H-phosphonate precursor, the other activator solution, driven by an infusion pump (Harvard Instruments), were used to deliver reagents to the channels. Mixing occurred as the reagents entered the channels through two neighbouring nozzles formed from 18 gauge syringe needles. After one minute, the solution was removed from the channels, the slides were rinsed with acetonitrile, the nucleotide precursors on the slide were detritylated in a staining jar containing 2.5% dichloroacetic acid in dichloromethane for 100 sec, and the slides rinsed again and dried in a stream of argon.

After the last synthesis cycle the slides were treated with iodine in tetrahydrofuran/pyridine/water for 4 min and with iodine in tetrahydrofuran/pyridine/triethylamine/water for 3 min to oxidise H-phosphonate linkages, and the heterocyclic bases were deprotected by incubation in 30% ammonia at 55°C overnight.

### Solvents for hybridisation

Tetramethylammonium chloride (TMACl, Aldrich) was dissolved in distilled water to give an approx. 5 M solution, charcoal (BDH) was added, the solution stirred and then filtered. After measuring the refractive index to determine the precise concentration (8), distilled water, SDS, Tris–HCl and EDTA were added to give a solution of the desired final concentration of TMACl containing 50 mM Tris–HCl (pH 8), 2 mM EDTA and 1 mg/ml SDS.

## RESULTS AND DISCUSSION

To test the influence of duplex length, base composition, hybridisation solvent and sequence complexity in a single simple

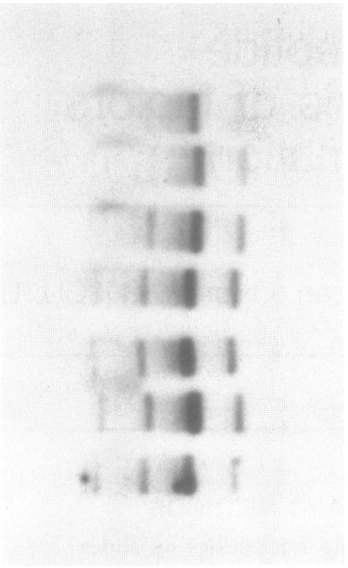

**Figure 1.** A typical time course experiment. On the autoradiograph seven strips cut from microscope slides are shown. The order of sequences on the strips is (from left to right): 3'- $G_6A_6$, 3'-$G_{12}$, 3'-$A_{12}$, and 3'- $A_6G_6$. Hybridisation was in 3 M TMACl for (from top to bottom) 5, 10, 15, 30, 60, 120 and 270 min. The probe used was a mixture of 5'-$T_{12}$, 5'-$C_{12}$, 5'-$C_6T_6$, and 5'-$T_6C_6$.

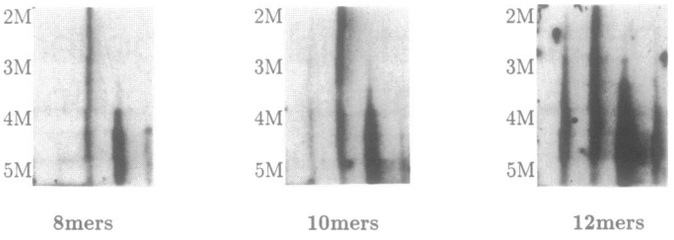


**Figure 2.** The results of hybridisations in chaotropic solvents of different concentration. On every autoradiograph seven strips cut from microscope slides and aligned in parallel are shown. Every strip was used for hybridisation in a different concentration of TMACl. The TMACl concentrations (from top to bottom) were 2 M, 2.5 M, 3M, 3.5 M, 4 M, 4.5 M, and 5 M. The order of sequences on the strips is (from left to right): 3'-$G_{n/2}A_{n/2}$, 3'-$G_n$, 3'-$A_n$, and 3'-$A_{n/2}G_{n/2}$, with $n$ = 8, 10, 12. The probe used in the hybridisation was a mixture of 5'-$T_{12}$, 5'-$C_{12}$, 5'-$C_6T_6$, and 5'-$T_6C_6$.

system, microscope slides were derivatised with the sequences 3'-$G_{n/2}A_{n/2}$, 3'-$G_n$, 3'-$A_n$, and 3'-$A_{n/2}G_{n/2}$, in four different, parallel lines on the same slide. On four different slides the length n was 6, 8, 10 and 12, respectively (Figure 2).

Oligopurine sequences were chosen because they lack self-complementarity which would introduce complex side effects and complicate the analysis (9). Four different hybridisation probes, $C_{12}$, $T_{12}$, $C_6T_6$ and $T_6C_6$, were synthesised and labelled with polynucleotide kinase using $\gamma^{32}$P-ATP (10). Uniformity of synthesis yields was tested by hybridising a mixture of all four labelled probes to the whole slide in a perspex container (set-up detailed in accompanying paper). Each slide was cut into seven strips 0.5 cm wide that corresponded to the area on the slide where a uniform hybridisation signal was obtained. Hybridisations were performed by placing each strip in a small Perspex container that required 330 $\mu$l of hybridisation solution to cover the whole surface. Prehybridisation is not necessary.

## Time course experiment

A typical time course experiment for oligonucleotide hybridisations was carried out as follows for the 12mer slide: The hybridisation solution (330 $\mu$l 3 M TMACl) included a mixture of 20,000 c.p.m. of each of the four probe oligonucleotides (between 45 and 150 fmol). Hybridisation was at room temperature (18°C). Seven strips were placed into containers for increasing lengths of time (for 5, 10, 15, 30, 60, 120 and 270 min), then removed, rinsed in 3 M TMACl and exposed for 12 hrs with intensification (Figure 1). The autoradiograph was scanned on an Optronics P-1000 drum scanner and the intensity values for individual duplexes measured using the Geltool software developed by J.K.Elder.

For sequences $G_{12}$ and $A_{12}$ there was a marked increase in hybrid yield up to 120 min of incubation, and for $G_6A_6$ up to 30 min, with generally lower yields after 270 min of incubation. The signal from $A_6G_6$ was too low for reliable measurement.

**Table I.** Intensity values for duplexes formed in hybridisations to the 8-, 10- and 12mer slides. Intensity values were derived from a contour plot after subtraction of the background stemming from the film sheet. Absolute intensity values are listed.

| Conc. | n | $G_{n/2}A_{n/2}$ | $G_n$ | $A_n$ | $A_{n/2}G_{n/2}$ |
|---|---|---|---|---|---|
| 2 M | 8 | 0 | 40 | 0 | 0 |
| | 10 | 0 | 70 | 0 | 0 |
| | 12 | 10 | 60 | 0 | 0 |
| 2.5 M | 8 | 0 | 40 | 0 | 0 |
| | 10 | 0 | 50 | 0 | 0 |
| | 12 | 30 | 60 | 10 | 0 |
| 3 M | 8 | 0 | 40 | 10 | 0 |
| | 10 | 10 | 50 | 10 | 0 |
| | 12 | 40 | 70 | 30 | 10 |
| 3.5 M | 8 | 5 | 40 | 10 | 0 |
| | 10 | 10 | 30 | 20 | 10 |
| | 12 | 50 | 70 | 90 | 30 |
| 4 M | 8 | 10 | 45 | 70 | 10 |
| | 10 | 10 | 50 | 90 | 10 |
| | 12 | 70 | 70 | 120 | 60 |
| 4.5 M | 8 | 10 | 50 | 110 | 30 |
| | 10 | 10 | 40 | 110 | 20 |
| | 12 | 70 | 60 | 160 | 90 |
| 5 M | 8 | 10 | 30 | 105 | 30 |
| | 10 | 10 | 30 | 130 | 30 |
| | 12 | 40 | 30 | 130 | 30 |

This result suggested that analysis of factors affecting reate should be carried out for 30 min or less, before thermodynamic equilibrium is reached.

## Factors influencing duplex yield

The hybridisation solutions (330 $\mu$l) were made in varying concentrations of TMACl (from 2 M to 5 M in increments of 0.5 M) and included a mixture of 20,000 c.p.m. of each of the four probe oligonucleotides (between 60 and 200 fmol).

Hybridisation was at room temperature (18°C) for 30 min. The slides were taken out of the containers, rinsed in a solution containing the corresponding concentration of TMACl, aligned parallel to each other in the order of ascending TMACl concentration, exposed for 13 hrs with intensification (Figure 2), the autoradiograph scanned and hybridisation signals quantified (Table I).

**Table 2.** Relative intensity values derived from the autoradiograph. Intensities are listed relative to the value for $G_n$ which remained fairly constant when increasing TMACl concentration.

| Conc. | n | $G_{n/2}A_{n/2}$ | $G_n$ | $A_n$ | $A_{n/2}G_{n/2}$ |
|---|---|---|---|---|---|
| 2 M | 8 | 0 | 100 | 0 | 0 |
| | 10 | 0 | 100 | 0 | 0 |
| | 12 | 17 | 100 | 0 | 0 |
| 2.5 M | 8 | 0 | 100 | 0 | 0 |
| | 10 | 0 | 100 | 0 | 0 |
| | 12 | 50 | 100 | 17 | 0 |
| 3 M | 8 | 0 | 100 | 25 | 0 |
| | 10 | 0 | 100 | 20 | 0 |
| | 12 | 57 | 100 | 17 | 14 |
| 3.5 M | 8 | 13 | 100 | 25 | 0 |
| | 10 | 33 | 100 | 67 | 33 |
| | 12 | 71 | 100 | 129 | 43 |
| 4 M | 8 | 22 | 100 | 156 | 22 |
| | 10 | 20 | 100 | 200 | 20 |
| | 12 | 100 | 100 | 171 | 86 |
| 4.5 M | 8 | 20 | 100 | 220 | 60 |
| | 10 | 25 | 100 | 275 | 50 |
| | 12 | 117 | 100 | 267 | 150 |
| 5 M | 8 | 33 | 100 | 350 | 100 |
| | 10 | 33 | 100 | 433 | 100 |
| | 12 | 133 | 100 | 433 | 167 |

*Effect of oligonucleotide length.* There was no hybridisation signal detectable on the 6mer slide, whereas signal was obtained on the 8mer slide for all $G_8$ duplexes. This suggests that 7 to 8 nucleotides are required for duplex formation under the conditions chosen.

The intensity of hybridisation of the $G_n$ lines remain fairly constant for n = 8, 10, 12 for all TMACl concentrations (as expected from the postulated mode of action of the $TMA^+$ [11, 12]), and so these oligonucleotides were chosen for analysis of the dependence of duplex yields on length.

A simple theory predicts the relative amounts of duplex formation. Let $m$ be the minimum length required to form a duplex, and let $n_p$ and $n_t$ be the lengths of probe oligonucleotide in solution and target oligonucleotide on the support, respectively. For homopolymers there should be

$$n_p + n_t - 2m + 1$$

possibilities of stable duplex formation. For a length of $m = 7$ this would lead to a ratio of 11:9:7 for oligonucleotide duplex formation with target 12-, 10- and 8mers. The average intensities for $G_{12}$, $G_{10}$ and $G_8$ (omitting the 5 M experiment for $G_{12}$ and the 3.5 M experiment for $G_{10}$ that show marked decreases in duplex yield when compared with their neighbours, Figure 2) were 65:48:41, equivalent to 11.1:8.2:7, which is in very good agreement with theory. Thus for sequences $G_n$ the expected dependence of duplex formation on oligonucleotide length was observed.

For other sequences the results are less easy to interpret. The $A_n$ sequences show ratios that differ markedly with TMACl concentration (Table I). For all TMACl concentrations the intensity order is $A_{12} > A_{10} > A_8$, as expected, but the relative intensities change from 21:7:7 (3 M TMACl) to 8.7:8.7:7 (5 M TMACl), i.e. the difference between 8, 10 and 12 base pairs decreases with an increase in TMACl.

For sequences $G_{n/2}A_{n/2}$ and $A_{n/2}G_{n/2}$, where only one duplex formation in register is possible for every length, duplex yield increased with $n$, reflecting the greater stability of longer duplexes (slower 'back reaction') and also faster kinetics due to increased opportunities for nucleation ($\sqrt{n}$ dependence [13]) with an increase in length.

*Effect of TMACl concentration.* The signals from the $G_n$ lanes vary little with TMACl concentration. It is therefore convenient to express measured intensity values relative to $G_n$ (Table II). (This corrects for uniformly reduced yields in all sequences of the 5 M 12mer and 3.5M 10mer experiments.) For the three sequences $A_n$, $A_{n/2}G_{n/2}$ and $G_{n/2}A_{n/2}$ signal increased markedly with increasing TMACl concentration, despite the increase in viscosity (14). The effect was greatest with $A_n$. Taken together these results are consistent with the notion (11, 12) that the tetramethylammonium ion ($TMA^+$) binds preferentially to A:T base pairs and stabilises them.

*Effect of base composition.* Melchior and von Hippel (12) and Orosz and Wetmur (15) found that the $T_m$ in 3 M TMACl for long double-stranded DNA in carefully controlled melting experiments carried out at thermodynamic equilibrium was the same for DNA's with a GC content ranging from 31−72%. Wood *et al.* (8) and Jacobs *et al.* (3) extended these studies and showed a similar effect in 3 M TMACl for oligonucleotides with lengths down to 16mers and GC contents between 31 and 81%.

The present work shows that it is possible to achieve equal intensities for oligonucleotides of 100% AT and 100% GC content though the necessary concentration of TMACl depended on oligonucleotide length. For $G_{12}$ and $A_{12}$ equal intensity was achieved for a TMACl concentration between 3 and 3.5 M. For $G_n$ and $A_n$ in the 10mer and 8mer experiments similar intensities were obtained at a concentration between 3.5 and 4 M. This suggests that the shorter the oligonucleotide on the solid support the higher a concentration of TMACl is needed to equalise the contributions of A:T and G:C base pairs.

Under conditions which equalise the signal for $G_n$ and $A_n$, the sequences $G_{n/2}A_{n/2}$ and $A_{n/2}G_{n/2}$ were markedly weaker (Table II). A plausible explanation would be reduction in yield due to the effects of sequence complexity on reassociation rate, discussed below. This is supported by the fact that the intensities of $A_{n/2}G_{n/2}$ and $G_{n/2}A_{n/2}$ are similar.

*Effect of sequence complexity.* The choice of length for probe (12mers) and target oligonucleotides (6, 8, 10, 12) allowed to address another feature of oligonucleotide hybridisation, i.e. probe 'complexity' (13).

According to the model for reassociation presented above, whereas homoduplexes can form in several registers only one duplex formation in register is possible for the non homopolymeric probes $C_6T_6$ and $T_6C_6$. According to the Wetmur and Davidson theory of duplex formation (13), the rate limiting nucleation step should lead to lower yields for complex sequences relative to the homoduplexes, as is indeed the case. The simple model predicts ratios of 11:1, 9:1 and 7:1 for homo-oligonucleotides : mixed oligonucleotides in the 12mer, 10mer and 8mer experiments, respectively, if only the forward reaction is important in determining yields in simple salts.

On the other hand, if mainly the stabilisation of A:T base pairs determined duplex formation, for example by slowing down the back reaction (i.e. duplex disruption) for AT rich duplexes, the expected order of intensities would be $G_n > G_{n/2}A_{n/2}$, $A_{n/2}G_{n/2} > A_n$ for concentrations < 3 M, and $A_n > G_{n/2}A_{n/2}$, $A_{n/2}G_{n/2} > G_n$ for concentrations > 3.5 M.

The results obtained (Table II) suggest that both factors determine duplex formation under the chosen experimental conditions. For example, high concentrations of TMACl lead to yields of sequences $A_6G_6$ and $G_6A_6$ higher than $G_{12}$, i.e. stabilisation of duplexes by $TMA^+$ outweighs the kinetic advantage of a homo-oligomer. For lower concentrations of TMACl and for shorter lengths of mixed oligopurines the obtained intensities are considerably lower than for homo-oligomers, albeit not to the extent predicted by the simple kinetic theory.

## CONCLUSIONS

The experiments presented above illustrate the potential of a new simple system for the analysis of oligonucleotide–oligonucleotide interactions. In this example only four different sequences were synthesised on a glass slide.

One major advantage of the flat plate format is the possibility to obtain an image that shows up artefacts and experimental problems, not detected in the standard column set-up where scintillation counting or optical density measurements are used to quantify results. The possibility to analyse multiple probes in a single experiment under identical conditions, i.e. temperature, time, and amount of bound oligonucleotide, is a further advantage.

The experiments can be extended considerably. Saturating the oligonucleotides on the slides first with copious amounts of probe oligonucleotides at lower temperature and subsequent elution at successively higher temperatures allows to obtain melting curves and to calculate melting parameters., e.g. dissociation rate constants, as will be detailed elsewhere (9).

An extensive study on the behaviour of oligonucleotides in a variety of chaotropic solvents has recently been published [16]. The authors address the potential of different anions and guanidinium salts to modulate the hybridisation and melting properties of duplexes formed on solid supports and find that the $T_d$ for the same duplex differs by up to 90°C thus allowing for a wide range of hybridisation conditions. The study presented here is complementary in that a chaotropic cation was used in hybridisation solutions, but all other chaotropes could easily be used in the same way.

A similarly convenient system was recently reported by Zhang *et al.* (6) who attach oligonucleotides as spots on membranes after oligonucleotide synthesis and attachment of a spacer arm. The necessary reagent is as yet not commercially available.

## ACKNOWLEDGEMENTS

We would like to thank J.K.Elder for analysing the scans. U.M. was supported by the Maximilianeum, Munich, Germany, the Studienstiftung des deutschen Volkes, the Bayerische Begabtenförderung, and the Deutscher Akademischer Austauschdienst.

## REFERENCES

1. Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K. B. and Erlich.H.A. (1988) *Science* **239**, 487–491.
2. Smith,M. (1983) In Weissman.,S.M. (ed.), *Methods of DNA and RNA Sequencing*. Praeger Publishers, New York, pp. 23–68.
3. Jacobs,K.A., Rudersdorf,R., Neill,S.D., Dougherty,J.P., Brown,E.L. and Fritsch,E.F. (1988) *Nucleic Acids Res.* **16**, 4637–4650.
4. Astell,C.R. and Smith,M. (1972) *Biochemistry* **11**, 4114–4120.
5. Saiki,R.K., Walsh,P.S., Levenson,C.H. and Erlich,H.A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6230–6234.
6. Zhang,Y., Coyne,M.Y., Will,S.G., Levenson,C.H. and Kawasaki,E.S. (1991) *Nucleic Acids Res.* **19**, 3929–3933.
7. Andrus,A., Efcavitch, J.W., McBride,L.J. and Giusti,B. (1988) *Tetrahedron Lett.* **29**, 861–864.
8. Wood.W.I., Gitschier,J., Laskey,L.A. and Lawn,R.M. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1585–1588.
9. Maskos,U. and Southern.E.M. (manuscript in preparation).
10. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbor, New York.
11. Shapiro,J.T., Stannard,B.S. and Felsenfeld,G. (1969) *Biochemistry* **8**, 3233–3241.
12. Melchior,W.B.,Jr and von Hippel,P.H. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 298–302.
13. Wetmur,J.G. and Davidson,N. (1968) *J. Mol. Biol.* **31**, 349–370.
14. Chang,C.-T., Hain,T.C., Hutton,J.R. and Wetmur,J.G. (1974) *Biopolymers* **13**, 1847–1858.
15. Orosz,J.M. and Wetmur,J.G. (1977) *Biopolymers* **16**, 1183–1199.
16. Van Ness,J. and Chen,L. (1991) *Nucleic Acids Res.* **19**, 5143–5151.